Short communication: Proteins from circulating exosomes represent metabolic state in transition dairy cows

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ABSTRACT

Biomarkers that identify prepathological disease could enhance preventive management, improve animal health and productivity, and reduce costs. Circulating extracellular vesicles, particularly exosomes, are considered to be long-distance, intercellular communication systems in human medicine. Exosomes provide tissue-specific messages of functional state and can alter the cellular activity of recipient tissues through their protein and microRNA content. We hypothesized that exosomes circulating in the blood of cows during early lactation would contain proteins representative of the metabolic state of important tissues, such as liver, which play integral roles in regulating the physiology of cows postpartum. From a total of 150 cows of known metabolic phenotype, 10 cows were selected with high ($n = 5$; high risk) and low ($n = 5$; low risk) concentrations of nonesterified fatty acids, β -hydroxybutyrate, and liver triacylglycerol during wk 1 and 2 after calving. Exosomes were extracted from blood on the day of calving (d 0) and postcalving at wk 1 and wk 4, and their protein composition was determined by mass spectroscopy. Extracellular vesicle protein concentration and the number of exosome vesicles were not affected by risk category; however, the exosome protein cargo differed between the groups, with proteins at each time point identified as being unique to the high- and low-risk groups. The proteins α -2 macroglobulin, fibrinogen, and oncoprotein-induced transcript 3 were unique to the high-risk cows on d 0 and have been associated with metabolic syndrome and liver function in humans. Their presence may indicate a more

severe inflammatory state and a greater degree of liver dysfunction in the high-risk cows than in the low-risk cows, consistent with the high-risk cows' greater plasma β -hydroxybutyrate and liver triacylglycerol concentrations. The commonly shared proteins and those unique to the low-risk category indicate a role for exosomes in immune function. The data provide preliminary evidence of a potential role for exosomes in the immune function in transition dairy cows and exosomal protein cargo as biomarkers of metabolic state.

Key words: exosome, metabolic state, lactation extracellular vesicle

Short Communication

During the peripartum period, cows are at greater risk of metabolic and infectious disease. This is evident by an increase in the incidence of health events; an estimated 50% of cows experience at least one disease during the transition period (LeBlanc, 2010). Identification of individuals at risk of metabolic dysfunction could provide potential for preventive intervention (Ingvarsen et al., 2003; Halasa et al., 2007).

Exosomes are small, membrane-bound extracellular vesicles (ECV; 40–100 nm in diameter) exocytosed by cells. They contain components from the propagating cell (Simpson et al., 2009; Mathivanan et al., 2010), including proteins (Looze et al., 2009), lipids (Subra et al., 2007), and various nucleic acids (Gallo et al., 2012), and are hypothesized to regulate the function of target cells throughout the body (Denzler et al., 2000). Aberrant cellular function (particularly pathologic conditions) can alter the rate of release of ECV (Kim et al., 2003; Baran et al., 2010) as well as their protein composition, such that the contents reflect the physiological state of the originating cell (Mathivanan et al., 2010). Furthermore, as ECV are transported in blood

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as long-distance, intercellular messengers, they are potential biomarkers of disease state (Distler et al., 2006; Taylor and Gercel-Taylor, 2008). For example, tumor-derived exosomes contain different protein contents compared with those secreted from nonmalignant cells (Andre et al., 2002). Furthermore, proteomic investigation of bovine milk exosomes has revealed that these nanoparticles are important for cellular communication in bovines during lactation, particularly for lactation physiology and mammary immune function (Reinhardt et al., 2012, 2013).

Due to the reported link between disease pathogenesis and circulating ECV proteins, we hypothesized that exosomes isolated from cows at risk of metabolic dysfunction during the transition period will exhibit an altered protein composition and, if so, could be useful biomarkers of health. In the current study, exosomes were isolated from the plasma of 2 cohorts of cows identified as being either a low or high risk for metabolic dysfunction postcalving, and their protein content characterized using mass spectroscopy.

The Ruakura Animal Ethics Committee (Hamilton, New Zealand) approved all animal manipulations (RAEC#12799) in accordance with the New Zealand Animal Welfare Act (1999). The experiment was undertaken at Scott Farm, Hamilton, New Zealand (37°46'S 175°18'E) between July and October 2013.

Cows used in this study were part of a larger experiment ($n = 150$; Roche et al., 2015). From this larger group, 10 cows were identified as being at either high risk (**HR**; $n = 5$) or low risk (**LR**; $n = 5$) of metabolic dysfunction based on blood plasma nonesterified fatty acid (**NEFA**; mmol/L) and BHB (mmol/L), and liver triacylglycerol (**TAG**; % of liver wet weight) after calving. The HR group cows exhibited higher ($P < 0.01$) mean ($\pm$ SD) values for the 3 variables than those in the LR group during wk 1 ($d 4 \pm 2$) and wk 2 ($d 11 \pm 2$) postcalving: 1.80 versus 0.66 ± 0.32 , 1.00 versus 0.45 ± 0.25 , and 9.0 versus 1.8 ± 1.59 , for NEFA, BHB, and TAG respectively, during wk 1 postcalving, and 1.20 versus 0.30 ± 0.33 , 0.86 versus 0.48 ± 0.11 , and 10.3 versus 1.4 ± 1.59 , for NEFA, BHB, and TAG, respectively, during wk 2. Average milk yield ($P < 0.05$), fat percentage ($P = 0.2$), and protein percentage ($P < 0.05$) during wk 1 and 2 of lactation for the HR and LR groups were 23.6 kg, 5.0%, and 3.9%, and 18.4 kg, 4.2%, and 4.2%, respectively.

Blood samples for metabolite analysis (wk 1 and 2) and exosome extraction (d 0, wk 1, and wk 4) were collected by coccygeal venipuncture into evacuated blood tubes containing either heparin or EDTA anticoagulant, respectively. Blood was placed immediately on ice and centrifuged at $1,500 \times g$ for 12 min at 4°C; then, the plasma was aspirated and stored at -20°C until

assayed. Approximately 1 g (wet weight) of liver tissue was collected (wk 1 and 2) for TAG analysis as outlined by Lucy et al. (2009). Collected liver samples were snap-frozen in liquid nitrogen and stored at -80°C.

Metabolite analysis and liver TAG assays were undertaken by Gribbles Veterinary Pathology Ltd. (Hamilton, New Zealand). Plasma BHB (mmol/L) was assayed with a Roche reagent kit using colorimetric techniques on a Hitachi Modular P800 analyzer at 37°C (Roche Diagnostics). Plasma NEFA concentrations (mmol/L) were measured using Wako Chemicals kit (Osaka, Japan). Liver TAG content was analyzed using the Wako LabAssay Triglyceride kit (290-63701, Wako Chemicals USA Inc., Richmond, VA). For detailed analysis, refer to Roche et al. (2015).

Exosomes were isolated from plasma (1 mL) at d 0, wk 1, and wk 4 by ultracentrifugation as previously described (Salomon et al., 2014a; Sarker et al., 2014). In brief, plasma was diluted with an equal volume of PBS (pH 7.4) and centrifuged at $2,000 \times g$ for 30 min at 4°C; then, the supernatant was removed and centrifuged at $12,000 \times g$ for 45 min at 4°C (Sorvall, high-speed microcentrifuge, fixed rotor angle: 900, Thermo Fisher Scientific Inc., Asheville, NC). The resulting supernatant was transferred to an ultracentrifuge tube and centrifuged at $100,000 \times g$ for 75 min. The pellet was resuspended in PBS (30 mL), filtered through a 0.22- μ m filter, and then centrifuged at $100,000 \times g$ for 75 min. The resulting pellet was resuspended in PBS (500 μ L), layered on a discontinuous iodixanol gradient, and 12 fractions were collected, diluted with PBS, and centrifuged at $100,000 \times g$ for 2 h at 4°C. Finally, the pellet containing the enriched exosome population was resuspended in 50 μ L of PBS. After isolation, exosomes were characterized by the size distribution, presence of endocytic marker (i.e., TSG101 and CD63), and morphology by nanoparticle tracking analysis, Western blot, and electron microscopy, respectively. Concentration of ECV was determined by using the DC Protein Assay (Bio-Rad, Hercules, CA). To ascertain the number of exosomal vesicles (**NEV**) per milliliter of plasma, a commercially available kit was used following manufacturer's instructions (ExoEL-CD63A-1; ExoELISA kit, System Biosciences, Mountain View, CA). Briefly, exosome concentration was determined as total immunoreactive exosomal CD63, and NEV per milliliter was calculated using ECV concentration.

To determine protein composition within exosomes, a modified version of the method described by Salomon et al. (2014b) was used. Isolated exosomes from plasma were sonicated for 15 min and samples were diluted in 50 mM ammonium bicarbonate, pH 8.5, and reduced with dithiothreitol for 1 h. Proteins were then alkylated in 10 mM iodoacetic acid for 1 h in the dark.

The sample was diluted 1:10 with 50 mM ammonium bicarbonate and digested with trypsin (1 μ g of trypsin:20 μ g of protein; Promega, Madison, WI) at 37°C for 18 h. The samples were then desalted by solid-phase extraction using a STAGE tip protocol [stop and go extraction tips for matrix-assisted laser desorption/ionization, nano-electrospray, and liquid chromatography (LC)-MS sample pretreatment in proteomics]. The eluted peptides were dried by centrifugal evaporation to remove acetonitrile and redissolved in solution A (0.1% formic acid in water). The resulting peptide mixture was analyzed by LC-tandem MS (LC-MS/MS) on a Triple TOF 5600 System mass spectrometer (AB Sciex, Framingham, MA) equipped with an Eksigent Nanoflow binary gradient HPLC system (Eksigent, Redwood City, CA) and a nanospray III ion source. Digested peptides were separated using a ChromXP C18-CL TRAP cHiPLC (200 μ m $\times$ 6 mm, 3 μ m, 120 Å) and analytical cHiPLC columns (200 μ m $\times$ 15 cm; 3 μ m, 120 Å; Eksigent) using a linear gradient of 5 to 10% solvent B (acetonitrile/0.1% formic acid) for 2 min, 10 to 40% solvent B (58 min), 40 to 50% solvent B (10 min), 50 to 95% solvent B (10 min) with a flow rate of 500 nL/min. The column was flushed at 95% solvent B for 15 min and re-equilibrated with 5% solvent B for 6 min. The MS/MS spectra were collected using information dependent acquisition using a survey scan (m/z 300–2,000 with an accumulation time of 250 ms) followed by 12 data-dependent product ion scans of the 12 most intense precursor ions (m/z 100–2,000 with an accumulation time of 150 ms). The precursor selection criteria included a minimum intensity or 50 counts per second and a charge state greater than 2+. All mass spectra were analyzed using the Mascot and Protein Pilot search engines against the Swissprot database (<http://www.uniprot.org/>) with the species set as bovine. Positive identifications were ascribed where Mascot scores were greater than 30 (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2015-10786>). False discovery rate was estimated using a reversed sequence database.

To test the effects of risk category, week, and their interaction on protein concentration and NEV, a mixed model approach with repeated-measures ANOVA was used (PROC MIXED; SAS/STAT 12.1, SAS Institute Inc., Cary, NC). This was followed by Tukey's *t*-test for pairwise comparisons. Exosome number data were $\log_{10}$ -transformed to achieve homogeneity of variance. Significance was declared if $P < 0.05$; results are presented as least squares means $\pm$ standard error of the difference (SED).

The plasma concentration of ECV protein and the NEV in cows categorized as having either a high risk or low risk of metabolic dysfunction during the transition

Figure 1. (A) Plasma concentration of total protein (μ g/mL) extracted from extracellular vesicles (ECV) and (B) number of exosomal vesicles (NEV) per milliliter of plasma from cows characterized as being at either a high risk ($n = 5$) or low risk ($n = 5$) of metabolic dysfunction during the transition period. Exosome number data were $\log_{10}$ -transformed before statistical analysis; back-transformed data are presented for ease of interpretation, and error bars denote $\pm$ SEM.

period are presented in Figure 1. On average, cows had 740 ± 279.7 μ g/mL of ECV protein in blood and $6.7 \pm 3.74 \times 10^{10}$ NEV/mL of plasma; these concentrations are similar to those measured in other species (from 2×10^9 to 9×10^{12} in humans, depending on stage of gestation; Salomon et al., 2014a). We detected no relationship ($R^2 = 0.001$) between ECV protein concentration and NEV ($P = 0.91$).

Neither protein concentration nor $\log_{10}$ NEV was affected by risk category ($P = 0.50$ and 0.91 , respectively) or week postcalving ($P = 0.28$ and 0.44 , respectively), and there was no interaction between risk category and week postcalving ($P = 0.42$ and 0.79 , respectively). Both ECV protein concentration and NEV reportedly increase in situations of hepatic steatosis and nonalcoholic steatohepatitis (Povero et al., 2014). The lack of a difference in either variable in our results was,

therefore, somewhat surprising, considering the large differences in circulating NEFA and liver TAG between the risk categories. This might have been due to limited statistical power, with only 5 animals per treatment; future investigations should involve more animals. However, Povero et al. (2014) highlighted that the effect of hepatic steatosis (fatty liver disease) on ECV was progressive, with evident differences in steatosis after 4 wk in a mouse model of diet-induced nonalcoholic fatty liver disease. The diet, high in fat and deficient in choline L-amino acid, failed to result in differences in ECV protein concentration, but further exposure to the diet produced both a greater degree of steatosis and a linear increase in ECV protein with the degenerative liver condition. These results indicate that nonsymptomatic fatty liver in early lactation dairy cows may not be sufficiently severe or of sufficient duration to elevate the concentration of ECV protein or NEV.

Although there was no evident difference in either ECV protein concentration or NEV between risk categories or days postcalving, we identified exosomal protein signatures unique to each risk category (Figure 2), suggesting that the exosomal cargo may vary with metabolic state during the transition period. Between 19 and 25 distinct proteins (depending on postcalving time point) were identified from between 44 and 72 individual accession numbers. Details of the exosomal proteins unique to risk categories, as well as those in common, are presented in Table 1. Of the proteins identified, 8 were unique to the blood exosomes extracted from HR cows on d 0, 10 were unique to cows in the LR category, and 5 were common to both categories (Figure 2). Unique proteins identified in each risk category at each postcalving time point over the transition period are presented in Figure 3. At wk 1 postcalving, 5 proteins were identified as unique to cows in the HR category, 15 to cows in the LR category, and 5 proteins were common to both groups. By wk 4 postcalving, only 4 proteins were unique to cows in the HR category, 12 to the LR category, and 3 proteins were commonly shared.

Several of the exosomal proteins on d 0 and wk 1 postcalving that were unique to cows categorized as being at a high risk of metabolic dysfunction are associated with metabolic syndrome in humans. For example, Feng et al. (2015) identified increased concentrations of α -2-macroglobulin in saliva in patients with type II diabetes. Similarly, elevated concentrations of fibrinogen in blood have been extensively associated with metabolic syndrome, cardiovascular disease, and type II diabetes (reviewed by Mertens and Van Gaal, 2002) and as a marker of inflammation in dairy cows (McSherry et al., 1970). Several parallels have been drawn between metabolic syndrome in humans and the biology

Figure 2. Graphical representation of unique proteins identified in plasma exosomes isolated from cows characterized as being at high risk or low risk of metabolic dysfunction during the transition period based on circulating concentrations of nonesterified fatty acids (NEFA) and BHB and liver triacylglycerol (TAG) at wk 1 and 2 postcalving. (A) Blood sampled on the day of calving (d 0); (B) blood sampled wk 1 postcalving; and (C) blood sampled wk 4 postcalving. Elements unique to high-risk cows are dark gray, those unique to low-risk cows are white, and common elements are light gray.

Table 1. List of unique proteins identified in plasma exosomes isolated from cows characterized as being at either a high risk ($n = 5$) or low risk ($n = 5$) of metabolic dysfunction on the basis of circulating concentrations of nonesterified fatty acids, BHB, and liver triacylglycerol during wk 1 and 2 postcalving¹

Accession	Day of calving (d 0)		Week 1 postcalving		Week 4 postcalving	
	Protein name	Accession	Protein name	Accession	Protein name	Accession
Unique to high-risk cows						
R9QSM8	Alpha-2-macroglobulin	F6PMQ1	Coiled-coil domain containing 84	F1N2J5	Hyaluronan and proteoglycan link protein 4	
B8Y9T0	Fibronectin	E1B9N6	Envoplakin	G5E5H2	Immunoglobulin	
A5PJE3	Fibrinogen α chain	F1MB40	Zinc finger protein 320	G3N0V2	Keratin	
F1MAV0	Fibrinogen β chain	E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing	E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing	
F1MGU7	Fibrinogen gamma-B chain	F1MM36	Nuclear receptor coactivator 5			
Q3SYR8	Immunoglobulin J chain					
G3X690	Kinesin-like protein					
F1MH22	Oncoprotein-induced transcript 3					
Unique to low-risk cows						
F1MRD0	Actin, cytoplasmic 1	Q3SZG7	60S ribosomal protein L13	B0JYQ0	Albumin	
B0JYQ0	Albumin	B0JYQ0	Albumin	Q5GN72	Alpha-1-acid glycoprotein	
B0JYN6	Alpha-2-HS-glycoprotein	G3MZX1	C6 orf 132	R9QSM8	Alpha-2-macroglobulin	
F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1	F1N4V6	ELAV-like protein	F1MS24	Choroideremia (Rab escort protein 1)	
G3MYD7	Histone H3	F1MIY4	Growth arrest-specific 2 like 3	F1N2I0	Choroideremia-like (Rab escort protein 2)	
A5D792	Histone H4	E1BLD1	HECT domain containing E3 ubiquitin ligase 1	F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1	
G3N0V2	Keratin	G3MYD7	Histone H3	A5PJE3	Fibrinogen α chain	
E1BMR0	NACC family member 2, BEN and BTB (POZ) domain containing	Q05FF0	Interferon gamma receptor 2	F1MAV0	Fibrinogen β chain	
G3MXX8	Transcriptional repressor scratch 1	F1MZT1	Leucine-rich repeat LG1 family, member 2	G3MYD7	Histone H3	
E1BHT5	E3 ubiquitin-protein ligase UBR4	A6QNR4	Myotubularin related protein 4	A6QNR4	Myotubularin related protein 4	
		G5E560	Pleckstrin and Sec7 domain containing 1	E1B7S1	Poly (ADP-ribose) polymerase family, member 12	
		G3MZN6	Pleckstrin and Sec7 domain containing 2	E1BNF9	Signaling lymphocytic activation molecule family member 9	
		E1BNF9	Signaling lymphocytic activation molecule family member 9			
		E1B793	Serine/threonine kinase 36			
		E1B7F5	WD repeat and HMG-box DNA binding protein 1			
Common to both risk categories						
A5D7N2	Histone H2B	G5E5H2	Immunoglobulin	A5D7N2	Histone H2B	
G5E5I3	Immunoglobulin heavy constant mu	F1N6F5	Cytochrome P450, family 27, subfamily C, polypeptide 1	G5E5T5	Immunoglobulin heavy constant mu	
Q3T101	Immunoglobulin lambda	A5D7N2	Histone H2B	Q3T101	Immunoglobulin lambda	
E1BNF9	Signaling lymphocytic activation molecule family member 9	G5E5T5	Immunoglobulin heavy constant mu			
G5E5H2	Immunoglobulin	Q3T101	Immunoglobulin lambda			

¹Blood for exosome extraction was sampled on the day of calving (d 0) and at wk 1 and 4 postcalving.

of the postpartum dairy cow (Lucy et al., 2004). For example, chronic inflammation, the presence of insulin resistance, and the resultant increased concentration of NEFA (Chagas et al., 2007; Farney et al., 2013) create a natural link between the endocrine physiology of postpartum cows and the physiology of type II diabetic

states presented in humans (Harris, 2005; Lewis et al., 2002). It is not surprising that the exosome phenotype of the HR cows signified a degree of metabolic dysfunction. Cows in the HR category were chosen because their metabolic profile reflected an extreme tendency to mobilize adipose reserves (i.e., high NEFA), an inability of the liver to oxidize mobilized NEFA completely (i.e., high BHB), and a failure of the liver to export the resultant very low density lipoprotein to peripheral tissues (i.e., high liver TAG).

In addition to these markers of metabolic state, both envoplakin and oncoprotein-induced transcript-3 were elevated in the cows categorized by their metabolic profile as being at increased risk of disease. These proteins have been identified as being important in liver function, with envoplakin isolated from plasma in a human patient with hepatocellular carcinoma (Yokokura et al., 2006) and oncoprotein-induced transcript-3 identified as a protein specific to liver (Shen et al., 2009). It is unclear whether their presence in the exosomes of HR cows reflects metabolic dysfunction or is merely a signature of more metabolically active liver cells indicated by the greater uptake of NEFA and the accumulation of TAG. We propose that these proteins reflect the hepatic steatosis used to characterize these cows and, as such, may be useful biomarkers of a more severe pathological condition before clinical signs are evident.

Although identified by their metabolic profile as being at risk of disease, it is noteworthy that none of these animals succumbed to a clinical condition. This lack of progression to a disease state may also be reflected in their exosome profile. By wk 4, the proteins peculiar to the HR cows were no longer reflective of the metabolic syndrome phenotype, which is consistent with the timing of a return to normal physiological function; for example, the maximum rate of lipolysis has ended (McNamara and Hillers, 1986b; Roche et al., 2007, 2009), the process of lipogenesis has been reinstated (McNamara and Hillers, 1986a), the somatotrophic axis has recoupled (Lucy et al., 2009), and most measures of metabolic state and immune function have returned to near precalving baseline concentrations (Bauman and Currie, 1980; Heiser et al., 2015; Roche et al., 2015). Our results provide hints that exosomes could carry a signature of metabolic dysfunction, which, with appropriate characterization, could be used in lieu of a liver biopsy, for example. Further work is required to determine the sensitivity of the exosome protein cargo to the metabolic state of the periparturient cow and whether the signature could be evaluated from noninvasive samples, such as urine, milk, or saliva.

The proteins unique to the LR dairy cows and those in common between groups indicate a role for exosomes in immune function regulation. For example, signal-

Figure 3. Graphical representation of unique proteins at 3 time points: day of calving (d 0) and wk 1 and wk 4 postcalving in (A) high-risk (HR), and (B) low-risk (LR) cows.

ing lymphocytic activation molecule-9 is a protein involved in regulation of immune cell interactions and is required for normal development, homeostasis, and immune cell function (Volkova et al., 2014). Similarly, the commonality of immunoglobulin proteins is consistent with a role for exosomes in transporting important immune molecules between tissues. Furthermore, α -1-glycoprotein is an acute phase protein produced in response to inflammation (Hochepied et al., 2003). It has several immunoregulatory functions, including inhibition of inflammatory responses of several immune cells, such as lymphocytes and neutrophils, indicating a possible role for exosomes in regulating immune function in early lactation (Chiu et al., 1977; Theilgaard-Mönch et al., 2005).

In conclusion, although ECV protein concentration in blood and NEV did not differ between risk categories in the current experiment and, therefore, would not be suitable for identifying at-risk cows, the exosome protein cargo was different between cows categorized as being at either a high or low risk of metabolic dysfunction during the transition period. The proteins identified as unique to the HR cows were biologically consistent with the metabolic state by which cows were categorized. Further research will endeavor to improve the sensitivity of the LC-MS/MS protocol; however, these preliminary results indicate that exosomal protein cargo may have potential as a biomarker of subclinical disease. Furthermore, additional research is needed to determine if exosome signatures are sufficiently sensitive to identify at-risk cows likely to recover or those likely to suffer clinical disease.

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